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SUBJECT: Annual Report for Award Number **W81XWH-04-1-0828**

Title: Molecular Studies on MIC1/PDF in Human Prostate Cancer
(USAMRMC PC040502)

Principal Investigator: Batra, Surinder K., Ph.D.

Overview: *The overall objective of this proposal is to investigate the incidence, function and regulatory mechanism(s) of a highly expressed MIC-1/PDF gene in prostatic cancer cells.* We and other groups have observed that the macrophage inhibitory factor/prostate derived factor (MIC-1/PDF) is overexpressed during the progression of numerous cancer types including prostate cancer, few studies on oncogenic signaling cascades which might be involved in the regulation of this expression have yet been reported (1-6). Moreover, the mechanism of action of MIC-1 which appear to be dependent of cancer cell types is yet controversial (4,6). This is in part due to lack of information about the MIC-1 receptor or binding sites and intracellular signaling elements activated by this pleiotropic factor in cancer cells during prostate carcinogenesis. Therefore, this supports the importance of our work to further characterize the implication of MIC-1 expression and secretion in malignant transformation of prostatic epithelial cells.

During the funding period (one year), we evaluated for the first time, the possible implication of different oncogenic factors such as androgens, epidermal growth factor (EGF) and interleukin-6 (IL-6), which are recognized to contribute to the prostate cancer progression, in the regulation of the expression of secreted MIC-1 protein (Aim 2). Moreover, the functions of MIC-1 have been studied *in vitro* by using specific antibody directed against MIC-1 protein as well as by regulating its expression by using antisense and sense DNA constructs of MIC-1 protein on different prostatic cancer cell lines (Aim 1). We have published/submitted our work in three papers

The central hypothesis of this proposal is that highly-regulated and differentially-expressed MIC-1/PDF gene is associated with the acquisition of androgen-independent growth in prostatic tumors. The rationale for this hypothesis is based on the followings: I) identification of this gene via microarray analysis by our group using LNCaP model and others using normal verses tumor tissues from the prostate gland; II) incidence of high expression of MIC-1/PDF in prostate tumors compared to normal prostate tissues; III) regulation of MIC-1 expression by androgens that are known to be associated with the normal growth and malignant progression of prostate gland; and IV) structure of MIC-1/PDF protein that shows homology to TGF-beta superfamily, indicating its role in several unique biological functions. The immediate objectives of this proposed study are to define functions and regulatory mechanism(s) of MIC-1/PDF in prostatic tumor cells. The immediate objectives of this proposed study are to define functions and regulatory mechanism(s) of MIC-1/PDF in prostatic tumor cells.

A) PROGRESS REPORT

AIM 1: Investigate if MIC-1/PDF expression contributes to the tumor growth, androgen independence, and metastatic properties in prostatic cancer cells by 'overexpression' and 'knockdown' of the MIC-1/PDF gene. Cell lines that overexpress or null MIC-1/PDF will be analyzed for alteration in cell growth, morphological changes, and androgen responsiveness.

The LNCaPC-33 cells (MIC-1/PDF low expressing) was transfected with plasmid DNA (with and without MIC-1/PDF cDNA constructs). The MIC-1/PDF cDNA construct was tagged with a DNA

sequence that encoded for a specific protein epitope FLAG, recognized by a known monoclonal antibody M2 (Sigma). The FLAG tag was inserted at the 3'-translated region of the MIC-1/PDF cDNA. A double stranded, synthetic oligonucleotide was designed to encode DYKDDDDK in the primer. In-frame insertion of the oligonucleotide sequence was verified by sequence analysis. The epitope-tagged MIC-1/PDF cDNA was placed under the control of the β -actin promoter in the pH β APR1-neo vector. This expression vector has previously been used for expression of growth factor receptors and mucin genes. As such, this vector yielded high levels of expression of the cDNA and easy selection of stable, expressing cell lines. Our initial expression analysis using this construct showed synthesis and secretion of an appropriate size MIC-1 protein (Fig 1).

The MIC-1/PDF expression was associated with increased growth rates compared to vector control (Fig. 2) and parental cells ($p < 0.05$, $n = 3$), and the population doubling time at the exponential growing phase was approximately 31, 24 and 29, 42 and 48 hours for clone 1B, 2B, 2H, parental LNCaP C-33 and vector-alone control cells, respectively. The cell growth was also analyzed by BrdU incorporation assay. The histogram shows that the level of BrdU incorporation in all three PDF stable subclones was significantly higher than in the vector-alone or parental LNCaP C-33 cells ($p < 0.05$, $n = 3$). These results were in contrast to the published results in the DU-145 prostate cancer cells, in which treatment of recombinant MIC-1 did not change proliferation rates.

In another approach, we down regulated the MIC-1/PDF expression in an aggressive MIC-1/PDF overexpressing LNCaP-LN3 cell line using the MIC-1/PDF-antisense sequences. The antisense sequence of the MIC-1 exon 1 (# AF008303) containing the 5'UTR and signal peptide sequence complementary to the portion of the native MIC-1/PDF RNA transcript (target sequence). This fragment was inserted at the *EcoRI/BamHI* multiple cloning site of the pCDNA-3.1 vector. Binding of the antisense can disrupt the function of the target sequence either by blocking its function (binding to its splice junction Exon 1/intron 1), promoting its degradation, or by altering the structure of the target sequence. Both antisense and control vector (negative control) constructs were prepared and sequenced. These constructs were transfected for stable expression into LNCaP-LN3 cells. The cells were selected on a medium containing 200 μ g/ml Zeocin. Single colonies were selected and expanded for analysis. Clones showing integration of vector sequences were further analyzed. LNCaP cells, in which MIC-1/PDF expression is downregulated (Figure 3), showed decreased growth rates and anchorage-dependent growth on soft agar. We are also preparing anti-sense and also RNAi construct in retroviral vector. These constructs are in the process of characterization.

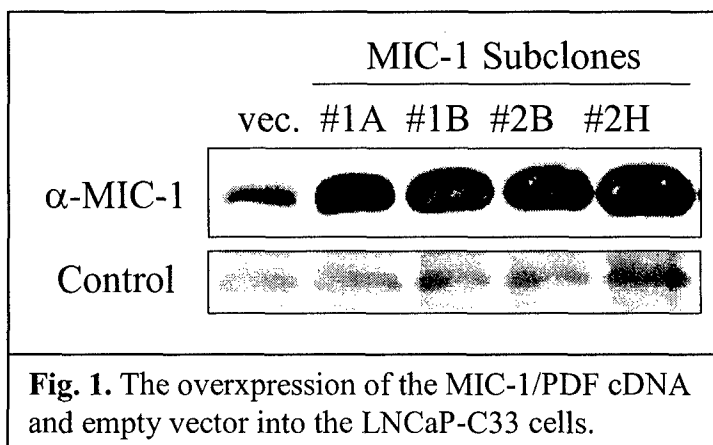


Fig. 1. The overexpression of the MIC-1/PDF cDNA and empty vector into the LNCaP-C33 cells.

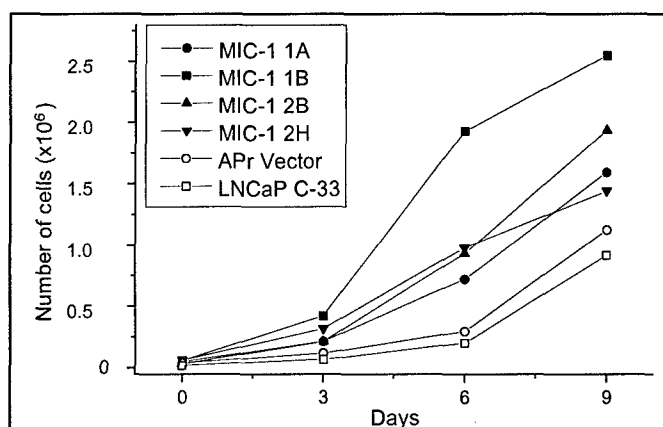


Fig. 2. In vitro growth kinetics of the LNCaP-C33 and its derived MIC-1/PDF

When these cells were transplanted in mice subcutaneously and followed for 60 days, metastasis to lymph node and liver was inhibited in anti-sense transfected cells compared to parent and vector transfected lines. Tumorigenicity was also lowered confirming the soft agar data

The results from our recent work carried out by MTT colorimetric tests and cytometric analyses by fluorescence-activated cell sorting (FACS) have indicated that the MIC-1 protein may contribute to the proliferative effects of certain of these growth factors. In particular, the serum- or EGF-stimulated growth of LNCaP and PC3 cells was significantly inhibited in the presence of specific antibody directed against MIC-1 protein. More specifically, the

inhibition of EGF-stimulated prostatic cancer cell growth induced by down-regulation of secreted MIC-1 protein was accompanied by increasing the cell population in the G1 phase of cellular cycle, concomitant with a reduction of prostatic cancer cell populations in the S and G2M phases. Similarly, the transfection of androgen-independent LNCaP-C81 cells with antisense MIC-1 DNA construct also inhibited their proliferation while the androgen-sensitive LNCaP-C33 cells that were transfected with sense MIC-1 construct, showed a higher rate of growth as compared to parental cells. However, the FACS analyses have revealed that the down-regulation or up-regulation of the MIC-1 expression in LNCaP cells did not significantly influence the rate of apoptotic death of these metastatic prostate cancer cells. Moreover, the estimation of the cell viability by trypan blue method indicated that the down-regulation or up-regulation of MIC-1 in LNCaP cells did not influence the rate of necrotic cell death. These works are being continuing by the characterization of the effect of down-regulation of MIC-1 protein on the invasiveness of PC cells by *in vitro* invasion assays. In fact, the invasive potential of prostate cancer cells is estimated by their ability to penetrate a Matrigel-invasion chamber whose method allowed an estimate of the metastatic potential of tumor cells *in vitro* (10).

Additional studies will be also carried out to determine the intracellular cascade elements which are activated by the secreted MIC-1 protein in different prostatic cancer cells after their stimulation by the aforementioned growth factors. In this matter, the microarray analyses will be performed by using new prostate cancer biomarkers oligoGEArray on the α -DHT and EGF-stimulated LNCaP and PC3 cells after treatment of these cells with the specific MIC-1 antibody or transfection with antisense MIC-1 construct. Moreover, the expression levels and activity of MAPK and PI3K/Akt cascade elements will be also estimated after down-regulation of the MIC-1 protein and compared to untreated or parental cell lines. This should allow to identifying the possible cascade(s) modulated by MIC-1 protein. Moreover, the effects of the down-regulation of MIC-1 in prostatic cancer cells will be also estimate in different animal models *in vivo* to determine its implication on the ability of the prostatic cancer cells to form the tumors and to metastasize at distant sites of prostate compartment.

AIM 2. Determine the mechanism(s) by which MIC-1/PDF expression is regulated in androgen-responsive and independent prostatic tumor cells. The LNCaP prostatic tumor cell model will be used

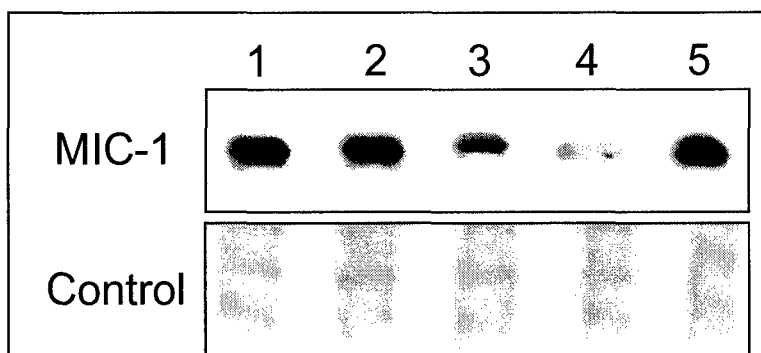


Fig 3. Down regulation of the MIC-1/PDF/PTGF-b secretory protein using anti-sense approach. Lane1: positive control (LNCaP-Ln3); lane 2: sense clone; lane 3 & 4: two different anti-sense clones; lane 5: vector control. (B) Lower panel showed the coomassie blue stained gel as a loading control.

to investigate these mechanisms because the MIC-1/PDF mRNA/protein can be turned 'on' and 'off' in vitro using culturing conditions in this model.

The results of our works carried out by RT-PCR and Western blot have revealed that the serum, dihydrotestosterone (α -DHT), EGF and IL-6 effectively induce an increase of the expression of secreted MIC-1 protein in androgen-sensitive LNCaP-C33 and androgen-independent LNCaP-C81 and PC3 cells (7). In contrast, the human PZ-HPV-7 nonmalignant prostate epithelial cells and androgen receptor (AR)-negative DU145 cells did not express the detectable levels of MIC-1 protein. The use of different specific inhibitors of intracellular cascades which are activated by these agents including AR antagonist, casodex, selective EGFR inhibitor, gefitinib, IP3K inhibitor, wortmannin and MEK inhibitor, PD98059 has allowed establishing their contribution in the MIC-1 expression. In particular, the results indicated that the stimulatory effect of these agents appears to be mediated in part *via* the androgen receptor (AR), MAPK and/or IP3K/AKT signaling cascades (Fig. 4 adapted from Mimeault, M and Batra, S.K.) (6). Additionally, we have also observed that the overexpression of AR in LNCaP-C33 was accompanied by an increase of MIC-1 expression and secretion. Altogether, these observations suggest that the MIC-1 expression might be regulated at least in part *via* the activation of AR, EGFR and IL-6 receptors during the prostate cancer progression to androgen-independent state.

In addition, we have observed that the basal level of MIC-1 expression which is observed in the absence of exogenous stimulatory agent was also significantly inhibited in presence of gefitinib. This

supports the fact that the activation of EGF-EGFR autocrine loop which contributes to prostate cancer progression (6,8-10), might play an important role in positive regulation of the MIC-1 protein expression in AR-expressing PC cells. Therefore, further works are being investigated to establishing the specific contribution of each one of these signaling pathways as well as on the possible synergistic effect induced by these growth factors on the transactivation of the MIC-1 promoter. In this matter, the use of MIC-1 promoter constructs should allow to identify the specific MIC-1 promoter elements involved in transactivation through these growth factors.

In parallel to these studies, the work is being carried out to develop monoclonal antibodies directed against the MIC-1 protein. The use of this antibody should allow to purifying the MIC-1 protein. Thus, the cross-linking studies will be performed with different cross-linking agents and

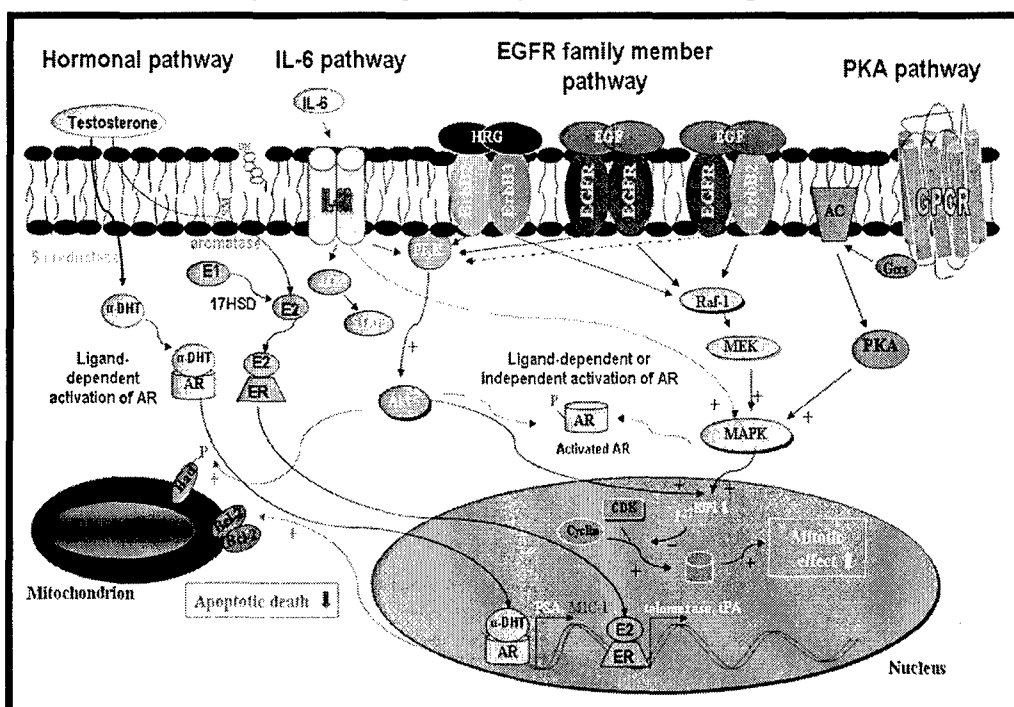


Fig. 4. A scheme showing mitogenic and anti-apoptotic cascades induced through AR, ER, EGFR family members, IL6, and PKA signaling pathways.

methods of photoaffinity labeling with the purified MIC-1 protein to identify the specific binding sites "receptors" in the PC cells.

AIM 3. *Analyze MIC1/PDF expression by immunohistochemistry and RT-PCR in prostate cancer tissues. This aim will provide co-relationship between MIC-1/PDF expression and histopathological grade of the tumors.*

To validate the clinical relevance of PDF expression in prostate cancer, we performed immunohistochemical staining of PDF in archival specimens. The intensity and the extent of staining in tissue sections, which contain both cancerous and non-cancerous cells, were analyzed. As reported previously in the preliminary data (2), expression of PDF was clearly seen in the cytoplasm of epithelial cells, while there was no specific staining in the fibromuscular stroma. The specificity of staining was further confirmed by pre-incubating the anti-PDF antiserum with the immunizing PDF peptide, which effectively competed out for the antibody staining (data not shown). The mean value of the composite score for PDF staining in benign glandular regions of 53 samples was 1.71 ± 0.16 ; while the staining in adenocarcinoma tissue on those same section slides had a composite score of 3.60 ± 0.35 . Statistical analyses clearly showed that PDF protein was significantly higher increased in prostate adenocarcinomas compared to the adjacent non-cancerous cells ($p < 0.001$, $n = 53$). The data thus suggest that PDF may play a role in prostate cancer progression. It is not possible to correlate the MIC-1/PDF immunostaining pattern with the tumor grade (Gleason grade) because I) most of the specimens ranged between 7 to 8 Gleason pattern. A large number of different grades of PCa are underway. We defiantly observed an enhanced pattern of MIC-1/PDF expression with the progression of the prostate tumor.

Perspectives

Hence, the aforementioned works are continuing to confirm whether the MIC-1 protein contributes to confer the most malignant phenotypes to cancer epithelial cells during prostate cancer progression. More specifically, the development of new pharmacological agents including monoclonal MIC-1 antibody which could be used to negatively regulate this biological effects *in vitro* and *in vivo* should shed the light on the possible oncogenic properties of MIC-1 protein.

Of therapeutic interest, the using of monoclonal MIC-1 antibody could also represent a promising strategy alone or in combination with other chemotherapeutic drugs for a more effective treatment of metastatic prostate cancer forms which are yet incurable by conventional treatments.

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B. PUBLICATIONS RESULTED FROM THIS GRANT

- 1) Mimeault M and **Batra S.K.** Recent advances on multiple tumorigenic cascades involved in prostatic cancer progression and targeting therapies. *Carcinogenesis*, in press, 2005
- 2) Singh A.P., Chauhan S.C., Bafna S., Johansson S.L, Lin, M.F. Moniaux N., and **Batra S.K.** Aberrant Expression of Transmembrane Mucins, MUC1 and MUC4, in Human Prostate Carcinomas. *Prostate*, in press, 2005
3. Chen, S.J., Karan, D., Johansson, S.L., Singh, A.P., Mimeault, M., **Batra, S.K.**, Lin, M.F. (2005) Prostate-Derived Factor (PDF) Promotes the Proliferation and Tumorigenicity of Androgen Receptor (AR)-Positive Prostate Cancer Cells (Manuscript near submission).